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Plant signaling pathways activating defence response and interfering mechanisms by pathogen effectors, protein decoys and bodyguards.

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Abstract.

Plants activate an immune response in defense against microbial pathogens. The first layer of immunity consists in the recognition of microbial fingerprints, called Pathogen Associated Molecular Pattern (PAMP), by a set of Pattern Recognition Receptors (PRR). In addition, the degradation products from fungi, bacteria and plant cells are recognised as Damage Associated Molecular Pattern (DAMP).

The first layer of plant defence is based on Pattern Recognition Receptors (PRR) on the membrane. These receptors, either receptor kinases or receptor-like proteins (RLPs), associating with cytoplasmic kinases, recognize the presence of PAMPs, thus activating a local response named PAMP-triggered immunity (PTI), that is not strong but effective towards many pathogen species. Here we discuss and focus on Elongation Factor Tu Receptors (EFR) and flagellin sensing (FLS) receptors. In leucine-rich repeat (LRR) receptor proteins, the hydrophobic LLR domains are exposed on external membranes, providing the protein–protein interaction modules. Plants evolved this protein-protein interaction domain several times during the development of mechanisms to defend themselves from viruses, virulence factors, enzymes and effectors of bacterial and fungal pathogens.

Pathogens in addition evolved proteins and enzymes that are injected in the plant cell to counterfight plant immune signaling pathways. These effectors are recognised by plant receptors sensing their presence of their cognate avirulence genes. These receptors originated from recombination during evolution and only occur in some specific tomato genotypes, instead of the widely occurring PPRs. Effector Triggered Immunity (ETI) allows a plant response to effector proteins that is more strong, but is race specific. It leads to local necrosis and apoptosis, and to the establishment of the hypersensitive response (HR). For biotrophic or hemibiotrophic pathogens, necrosis is an effective way to limit their spread, while for necrotrophic pathogens this is not efficient and sufficient way to limit their spread, since depends on the timing of infection and on the plant development phase. Pathogenic fungi strategy relies on the formation of specialised structures, or haustoria, that facilitate the nutrient uptake form plant cells. In this review we summarize the most recent knowledge on plant pathogens and the mechanisms they evolved to circumvent plant defences among which pathogen effectors, protein decoys inactivating plant defence signals. Effectors are recognised through their binding to plant proteins by means of

plant receptors, that activate the Effector Triggered Immunity (ETI). In particular, we focus on the Solanaceae, discussing general mechanisms and specific pathways that confer resistance to various pathogens.

There is an arm race between plants and fungal and bacterial pathogens that has led to new protein variants and protein decoys (pseudokinases, inhibitors and sponges blocking glucanases, and Transcription Activator Like Effectors). Advances in understanding the function of pathogen effectors will provide new ways to improve plant immunity and mechanisms of defence against their pests. Finally, we present possible combinations of interventions, from gene engineering to chemical priming, acting on signaling pathways regulated by jasmonate and salicylate hormones, to increase plant resistance and activate plant defences without affecting crop yields.

Introduction

The first layer of plant defence against pathogens consists in the recognition of microbial fingerprints, called Pathogen or Microbial Associated Molecular Pattern (PAMP/MAMP), by a set of Pattern Recognition Receptors (PRR). PAMP are classified as: 1) structural PAMPs that regroup molecules like polysaccharides (and lipopolysaccharides) involved in the maintenance of the microbial cell integrity [1-4] and 2) the encoded PAMPs that are made of amino acid sequences [5, 6]. Both PAMPs are under similar selective pressure from PRRs, but encoded PAMPs are under selection and evolve more rapidly, thanks to genome mutations. In addition to sequence conservation, encoded PAMP are spread in several pathogens, but not present in the plant hosts. For instance, the enigmatic MAMP of Xanthomonas (eMax) protein is present in several *Xanthomonads* [7], flagellin is present in motile bacteria, eubacterial Elongation Factor thermo-unstable (EF-Tu) is widespread [9, 10] and the necrosis and ethylene inducing peptide 1 (Nep1)-Like Proteins (NLPs) are present in several plant pathogen kingdoms (bacteria, fungi and oomycetes) [11].

Among proteins recognized as PAMP, the most studied in plant defense are flagellin and EF-Tu. EF-Tu, codified by the *tuf* gene, is one of the most abundant proteins in bacteria and belongs to the moonlighting protein family, i.e. proteins playing several functions carried by a single polypeptide chain. EF-Tu has also been found associated with bacterial membrane [12], thus allowing its recognition by plant membrane receptors [13].

Studying the minimal eliciting peptide in *Brassicaceae*, elf18, Zipfel identified the EF-Tu receptor (EFR), belonging to the Leucine Rich Repeat (LRR) receptors family. The conserved N-terminus has been shown to elicit innate immunity in Arabidopsis plants [14, 15]. EF-Tu may undergo N-terminal modifications having opposing effects. For instance, N-terminal acetylation enhances EF-Tu elicitor activity, whereas natural mutations within the 18 first amino acids of EF-Tu (elf18) lower the innate immune signaling [16]. Dicotyledonous plants (dicots) show differential responses to the K2R substitution in elf18. *Xanthomonas campestris* pv. *campestris* B100 produces an elf18B mutant while elf18G is present in *Pseudomonas syringae* pv. *tomato* DC3000, with lower activation of Hypersensitive Response (HR). *Solanaceae* plants lack a functional EFR, thereby relying on other PAMP sensing receptors.

Although monocotyledonous plants (monocots) lack elf18 recognition system [17], it has also been shown that a second and distinct EF-Tu epitope is able to induce immune responses in rice [18]: an EF-Tu middle region comprising Lys176 to Gly225, termed EFa50, is fully active as a PAMP in rice. In the leaves of rice plants, EF-Tu induced H_2O_2 generation and callose deposition, and also triggered resistance to co-infection with pathogenic bacteria.

Flagellin is recognized in plants by at least three flagellin receptors [8, 19, 20], specific to different plant lineages. Flagellin Sensing 2 (FLS2) is the receptor for the 22 amino acid peptide (flg22) derived from flagellin. Other flagellin receptors recognise longer peptides. FLS3 senses a 28 amino acid peptide derived from flagellin in tomato [19], while in rice an LRR receptor is able to recognise a flagellin C-terminal peptide [21-24]. Flagellin triggers cell death in tobacco thanks to bacterial O-glycosylation of the hypervariable part of flagellin [25, 26]. The flagellin C-terminal is glycosylated with several glycan repeats in *Acidovorax avenae* and *Pseudomonas syringae* pv. *tabaci* 6605.

An evasion mechanism is exemplified by the evolution of the flagellin-encoding genes in plant pathogens *Ralstonia solanacearum* or *Xanthomonas campestri pv campestris B186* (*XccB186*) to evade FLS2 recognition [27, 28].

A different evasion strategy is exemplified by the *Pseudomonads* AprA protein, which digests monomeric flagellin, thus hampering plant FLS recognition [29].

In plants, nucleotide-binding domain (NBD)- and leucine-rich repeat (LRR)-based receptors and receptor like proteins (RLPs), lacking the cytoplasmic kinase domain, are sentinels of plant immunity that monitor host proteins for perturbations induced by pathogen released proteins, able to trigger defence signals [5, 30]. In LRR receptors, the hydrophobic LLR domains are exposed on external protein surfaces, thus determining protein–protein interaction modules. Plants evolved this protein-protein interaction domain several times during the development of mechanisms to defend themselves from viruses, virulence factors, enzymes and effectors of bacterial and fungal pathogens. RLKs, once activated by their ligands, form a complex with their co-receptors, such as BRI-ASSOCIATED RECEPTOR KINASE 1/SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3 (BAK1/SERK3) [6, 7, 32-37], allowing trans-phosphorylation between BAK1 and FLS2 or EFR kinase domains. After flg22 binding, FLS2 releases BOTRYTIS-INDUCED KINASE 1 (BIK1) and associates with BAK1.

A GxxxGxxxG motif in the trans-membrane (TM) domain of LRR receptors and RLPs, is essential for interaction with SUPPRESSOR OF BIR1-1/EVERSHED (SOBIR1/EVR) [31]. LRR-RLPs constitutively interact with SOBIR1, with interplay of kinase activity and reciprocal phosphorylaton. Upon ligand perception by LRR-RLP, the associated SOBIR1 in turn interacts with BAK1/SERK3, suggesting that a similar downstream signalling pathway is activated (see scheme in figure 1). Peptide ligand receptor complex formation has been shown to follow a two step phase: flg22 first triggers RLK heterodimerization and later assembly into larger complexes through homomerization [36]. This event initiates downstream signalling for defence activation, followed by internalization of the activated PRR complexes through endocytosis, that poses an end to the signal allowing reconstitution of the receptors onto the membranes (see scheme in figure 2). The downstream signalling results in the activation of a plant response, including transcription of Pathogenesis-related (PR) proteins.

The signaling pathway that lead to plant defence involves the phosphorylation of LRR-receptors, their translocation from membranes to vacuoles, as a negative feedback, the activation of downstream Mitogen Activated Protein Kinase Kinases (MAPKKs). MAPKKs signaling is involved in plant defense, regulation of vesicle trafficking, activation of Transcription Factors (TFs), and transcription of target genes such as *AVR9/CF-9 RAPIDLY ELICITED 132* (*ACRE132*) and *HAIRPIN INDUCED 1* (*Hin1*) whose expression as defense-related marker genes denotes the efficacy of the treatment experiments.

The plant LRR-XII family, differentially expanded in rice and Arabidopsis, includes either FLS2 and Xa21 [37, 41]. PTI responses include the production of reactive oxygen species (ROS) [30],

callose deposition in the plant cell wall, stomatal closure and the activation of defense-related genes, and interfere with the survival and multiplication of non-adapted microbial invaders [38-40]. ROS are generated in the apoplast by the respiratory burst oxidase homologs (RBOHs) [40, 41], and the RLK signaling and ROS production are each influenced by the other (crosstalk).

The growth hormones jasmonic acid (JA); salicylic acid (SA); ethylene (ET), indole acetic acid (IAA); gibberellic acid (GA), activate signaling pathways that drive changes in gene expression, resulting in specific defense responses and induction of pathogenesis-related proteins. Defense comes at the cost of reduced growth, and plants have evolved strategies to minimize costs and optimize the balance between growth and defense [42]. Different cellular pathways, dependent on phytohormones-activated Transcription Factors, bring to the expression of defense proteins.

Jasmonate is known to regulate abiotic and biotic stress response: its active compound, 7-iso-jasmonic acid-isoleucine (JA-Ile), releases the JASMONATE-ZIM-DOMAIN (JAZ) repressor from the transcription factor MYC2, containing the G-box domain; JA also activates transcription factors involved in abiotic stress, that are ethylene and JA regulated, containing the GCC motif [87, 88].

The induction of defence proteins such as pathogenesis-related protein 1 (PR1) and pathogen-induced defensin (PDF1.2) marker genes has been extensively used as marker of plant defense regulated by SA and JA, respectively. The mon-expressor of pathogenesis-related genes1 (NPR1) is a well known master regulator of gene expression during immune response, a transcription activator sensing the redox state of the plant cell. NPR1 binds directly to SA via two cysteine residues. Upon SA treatment, NPR1 oligomers are monomerised due to a change in the intracellular redox status. NPR1 monomers are translocated to the nucleus where they activate gene expression [89].

The plant hypersensitive response (HR) leading to disease resistance is characterized by the rapid accumulation of nitric oxide (NO). Nitrosylation of cysteines in enzymes of JA synthesis have been found to be important in regulating JA signaling. In plants, NO-mediated nitrosylation activates transcription factors such as MYB (MYeloBlastosis gene), a basic helix-loop-helix (bHLH) TF, involved in JA-dependent signaling. SA binding protein 3 (SABP3), modulating the SA response and integrating the JA signaling, is nitrosylated by NO during the hypersensitive response (HR) [89].

This NO signaling triggers localized hypersensitive cell death, inducing sets of defence genes, and mediates a network that is involved in the establishment of Systemic Acquired Resistance (SAR). In general, local and systemic defense response, including systemic acquired resistance (SAR), against biotrophic pathogens is mediated by SA, whereas JA and ET mediate responses against necrotrophs. The crosstalk between SA and JA pathways can be either mutually antagonistic or synergistic.

Pathogen effectors: Avirulence genes

There are several mechanisms that pathogens use to switch off plant defense activation Pathogens secrete toxins and/or effector proteins able to hijack PTI signaling and to inactivate PRR-based defences, in order to allow nutrients availability, and to support pathogen spread. Large repertoires of effector activities have been found for pathogens with different lifestyles. There are effectors in extracellular bacteria released in host cells by type III secretion system (TTSS) (T3S); other effectors in oomycetes and fungi able to invaginate specialized feeding organelles, called haustoria, into host cells. The effectors are proteins or secondary metabolites that subvert host physiology for the advantage of the pathogens. The effector proteins are

delivered into the host plant to manipulate host defence in several ways, by protein post-translational modification, exerting a wide range of enzymatic modifications, or targeting host proteins to degradation, interfering with phytohormone signaling, vesicle transport and the formation of the cytoskeleton, and by nuclear localisation, acting as transcription factors modifying gene expression profiles. These effectors, named *Avirulence (Avr*) genes, or *Xop* genes for *Xanthomonas oryzae* pathogenesis genes, modify and inactivate a series of plant signaling pathways leading to a block in plant immune defences [43-48].

Effectors represent adaptation to hosts, evolved from genes and functions from saprotrophic ancestors and plant symbionts, from molecules used to suppress ecological competitors. Effectors from evolutionarily diverse pathogens are highly specialised and specific for a limited number of plant proteins with activity and role linked to plant immunity.

The effectors are recognised by plant receptors sensing their presence of their cognate avirulence genes. For instance, the receptors for *Cladosporium fulvum* (Cf) Avr effectors are RLPs that lead to the formation of protein complexes. The tomato SOBIR1 acts as a co-receptor for Cf proteins. These effectors have been numbered according to the sequential order of discovery.

The Cf receptors, originated from recombination during evolution, are present only in some specific tomato genotypes, leading to race-specific resistance and a strong Hypersensitive Response (HR). This leads to effector triggered immunity (ETI). It has been shown that PTI and ETI have similar anti-pathogen outputs: the effector-triggered immune response is stronger, but race specific, leading to a localised programmed cell death (PCD) or to necrosis, for the containment of pathogen spread, contributing to HR.

Botrytis and *Pythium* are necrotrophic pathogens, that destroy plant tissues with limited species specificity [49]. The pathogenicity is based on degrading enzymes or toxic metabolites, with a limited number of effectors produced, and cell killing protein toxins. Other fungi have a highly specialized life cycle and restricted host range. The fungi start a growth within the plant apoplast without any symptom, then pathogens produce metabolites and toxins targeting specifically gene products, i.e., a single gene of the pathogen interacts with a single gene of the plant to induce susceptibility [46]. Biotrophic pathogenic fungi, such as rust, powdery mildew, or white rust and downy mildew oomycetes, show host specificity and dependence on the host plant for metabolites. In this case, evolution toward pathogenicity has led to genome shrinking with loss of genes involved in nutrient acquisition, with expansion of effector genes [46].

To protect the effectors from host proteases, fungi evolved several mechanisms of protease inhibition [50]. Many effector proteins secreted into the apoplast are rich in cysteine residues forming cystine knots and disulfide bridges, that increase protein stability in a protease-rich environment, or have high affinity to plant proteases [50-57].

Many pathogen effectors are inhibitors of plant proteases [51]. The tomato cysteine proteases Rcr3, Pip1, aleurain, and TDI-65 are necessary during basal host defence against fungal pathogens. Pip1 and Rcr3 are strongly induced by fungal effectors and by hormones such as salicylic acid (SA) [51].

Cystatin-like EPIC proteins, secreted by the oomycete *Phytophthora infestans*, target the C14 proteases in *Solanaceae*. *P. infestans* (Pinf), during tomato infection produces EPIC1 and EPIC2b (effector protease inhibitor, cystatin-like), cysteine protease inhibitors that target two tomato proteases, C14 and *Phytophthora*-inhibited protease-1 (Pip1) [50, 51]. The *P. infestans* EPI1 and EPI10 protease inhibitors [52], induced during infection, interact and inhibit the P69B cysteine protease in tomato apoplast [51]. Oomycetes can produce up to 12–15 Kazal type serine

protease inhibitors [52]. In maize, fungal cysteine protease inhibitor Pit2 binds and inhibits CP2, CP1A and CP1B proteases. AvrP123, in *Melampsora lini*, is a Kazal-like proteinase inhibitor [53].

In Arabidopsis, pathogen *Hyaloperonospora arabidopsidis* (Hpa) produces cystatin-like EPIC inhibitors targeting RD21 cysteine protease. The *rd21* plant mutants were shown susceptible to *Botrytis cinerea* infection [54].

Effector proteins from *Ustilago maidis* can block plant immune responses by inhibiting the expression of cysteine proteinase C69 [55].

On the other side, pathogens relay on proteases for the digestion of plant tissues [69]. Therefore, plants acquired a large spectrum of proteinase inhibitors to fight and block the pathogen proteases. Protease inhibitors belonging to the Kunitz family are present in higher plants, such as Solanaceae. Potato, tomato and other Solanaceae contain various Kunitz-type protease inhibitors (PKPIs), with a size of 24.000 Dalton (Da) [68]. Potato tubers infected by *Aspergillus carbonarius* accumulated several inhibitors with specificity toward different proteases, such as trypsin/chymotrypsin inhibitors in the early phase of infection, followed by papain, ficin, bromelain and cathepsin B inhibitors in later stage of infection [68]. It may be possible that KPIs are processed, as the PKPI P58514.2 [69], a strong inhibitor of *P. infestans* infection.

In tomato, the Kunitz-type proteinase inhibitor 4 (KTI4), with size 21 kDa, functions downstream of the vacuolar protease *Sl*VPE3. The suppression of expression of VPE3, by gene silencing, affects fruit susceptibility to pathogen infection and fruit disease resistance. The susceptibility of tomato fruit to necrotrophic pathogens such as *Botrytis cinerea* increases during fruit ripening: KTI4 requires a processing by *Sl*VPE3 into smaller peptides, since their presence is related to tomato resistance to *B. cinerea* [67].

***Cladosporium fulvum* effectors: Avr2/Rcr3/Cf-2 system**

During infection, *C. fulvum* produces several effectors with protease inhibitor function. Both Rcr3 and Pip1 plant proteases are inhibited by Avr2 from *C. fulvum*. Avr2, being a cystatin, inhibits tomato cysteine proteases, including Rcr3, Pip1, aleurain, and TDI-65, important in basal host defence.

The binding of Avr2 to Rcr3 causes the recognition of the complex by tomato Cf-2 immune receptor [51]. When Avr2 binds to Rcr3, this interaction is sensed by Cf-2 leading to Effector Triggered Immunity (ETI).

Avr2 inhibits also *Arabidopsis* cysteine proteases. XCP2, RD21A and Responsive to Dehydration 21B (RD21B) were identified using yeast two-hybrid assays as interacting partners of protease inhibitors in Arabidopsis [56], that stabilise XCP2. In a biochemical study, XCP1, XCP2 and CPR1 showed high Avr2 affinity, while Responsive to Dehydration 21A (RD21A), aleurain and aleurain-like thiol proteases had low Avr2 affinity [57-60].

Rcr3, targeted by Avr2, is involved in basal defense and satisfies the definition of a pathogenesis-related (PR) protein [61].

The guard model hypothesis proposed by Jones and Dangl [65] requires that some R proteins monitor a pathogen effector target rather than interact directly with their cognate pathogen effector. If a pathogen effector mutates to enable modification of the guard-target without being detected by the guard, then the guard and guard-target complex come under evolutionary pressure to regain recognition capacity or avoid modification by the effector or both.

The Cf-2–Rcr3–Avr2 interaction is a well-characterized example of an interaction in the tomato–*C. fulvum* pathosystem that conforms to the guard hypothesis. Rcr3 has the hallmarks of

pathogen-driven positive selection. First, it belongs to a multigene family that resides in a complex locus with five paralogs, including Pip1, which is also targeted by Avr2 [66]. Second, there is evidence for divergent selection in and around the substrate-binding grooves in Rcr3 and Pip1 [53].

***Cladosporium fulvum*: ExtraCellular Proteins (ECPs) as effectors in Solanaceae infection**

AvrECP1, AvrECP2, AvrECP4, AvrECP5 and AvrECP7 are secreted cysteine-rich proteins. This property may confer increased resistance to proteolysis and highly compacted structure. AvrECP6 encodes a larger protein containing three LysM carbohydrate-binding domains that may bind chitin. To date, 11 different *ECP* and *Avr* genes have been cloned, and at least additional eight are predicted, based on distinct gene-for-gene interactions [62, 63].

Resistance genes conferring recognition of ECP1, ECP2, ECP4, and ECP5 have been identified from *L. pimpinellifolium* and were found to map to a cluster of *Homologs of Cladosporium-resistance gene Cf-9* (*Hcr9*) genes, located on the short arm of tomato chromosome 1.

Avr9B targets a basal defense protein that is significantly upregulated or only expressed in adult plants. Cf-9B recognizes a necrosis-inducing protein (NIP) present in the apoplast of *Nicotiana benthamiana (N. benthamiana)*. The necrosis-inducing protein in *N. benthamiana* corresponds to the protein targeted in tomato by Avr9B, the complex being recognised by Cf-9B. The heterologous expression of Cf-9B and the *Hcr9* genes *Peru1* and *Peru2* triggers necrosis in a number of *Nicotiana* species [63].

***Cladosporium fulvum* effectors: Avr9/Cf-9 system**

Avr9 is sensed by Cf-9. Avr9 in *C. fulvum* is a protease inhibitor with a cysteine-knot structure, resembling a carboxypeptidase inhibitor [56]. Avr9 is recognised by High Affinity Binding Sites (HABS) on plasma membrane, and this interaction is sensed by the LRR receptor Cf-9, triggering receptor activation and signaling.

In Solanaceae, the pattern of responses to Cf-9 alone or in combination with Avr9 is mirroring the response to Cf-4 alone or in combination with Avr4 [63]. Assuming that the Cf-4–Avr4 interaction is direct, it is deduced that also Cf-9–Avr9 interaction is direct, probably depending on the binding of Avr9 to HABS present in Solanaceae. A difference between Cf-9 and Cf-4 is found in lettuce, which responds to Cf-4/Avr4 interaction but not to Cf-9–Avr9 interaction. Presumably the failure of the Cf-9–Avr9 combination to do so can be attributed to the absence of the HABS in lettuce.

***Pseudomonas syringae* effector proteins**

Pseudomonas syringae employs a type III secretion system to inject 20-30 different type III effector (T3SE) proteins into plant host cells [70].

The *P. syringae* YopJ/HopZ superfamily of T3SEs has acetyltransferase activity. Acetylation of an NB-LRR plant immune-effector complex suppresses immunity.

HopAO1, secreted by *P. syringae*, is a tyrosine phosphatase that reduces EFR phosphorylation and prevents PTI (43, 46). XopE1 and XopE2 belong to the HopX (AvrPphE) family of putative transglutaminases with different enzymatic activities like proteases, peptide N-glycanases, and DNA repair proteins [43, 72].

Many plant receptors for avirulence genes are LRR proteins acting in concert with co-receptor kinases. The presence of pseudokinases devoid of activity interferes with effector function [73-

75]. There is a competition between the pseudokinase and the pathogen effector for its natural target, with a sponge effect.

The *Arabidopsis* Nucleotide-binding domain LRR (NLR) protein AtZAR1 (acronym for HOPZ-ACTIVATED RESISTANCE1) was shown to require the ZED1-RELATED KINASE (ZRK) ZRK3. ZED is the pseudokinase, acting as a complex formation hub. HopZ1a is an acetyltransferase that acetylates the pseudokinase AtZED1 and triggers recognition by AtZAR1.

HOPZ-ETI-DEFICIENT1 (AtZED1) is a receptor-like cytoplasmic protein that recognizes the *Pseudomonas syringae* (PtoDC3000) type III effector HopF2a. HopF2a does not directly ADP-ribosylate ZRK3: probably ZRK3 acts as an adaptor between AtZAR1 and an unidentified kinase that is modified by HopF2a. AtZAR1 is thus a recognition hub able to activate three LLR proteins (AtZED1, ZRK3, and RKS1) of the type XII Receptor family, to sense three T3S effectors that have different enzymatic activities and are from different bacteria [91].

AvrAC (XopAC*Xcc*) uridylylates BIK1 kinase, with inhibition of BIK1 phosphorylation. PBL2, a paralog of BIK1, is similarly uridylylated by AvrAC. However, in contrast to BIK1, PBL2 uridylylation is specifically required for host recognition of AvrAC to trigger immunity, but not AvrAC virulence. PBL2 thus acts as a decoy and enables AvrAC detection [72].

Among bacterial effectors that interfere with post-translational modifications, HopM1 interacts and induce degradation of an ADP-ribosylation factor-guanine nucleotide exchange factor (ARF-GEF) involved in vesicle trafficking. Some pathogen effectors such as HopU1, HopF2, and AvrRpmi1 are toxins belonging to cholera-like (C type) ADP Ribosyl Transferases (ART): HopU1 ADP ribosylates and inactivates GRP7 RNA binding protein; while HopF2 is a diphtheria-like (D type) ART that modifies MAPKKs. XopQ*Xoo* is present in complex with adenosine diphosphate ribose, thus mimicking a Macrodomain protein, thus possibly interfering with ADP ribose hydrolases or masking these post-translational modifications [43, 72].

Defensive effectors in plant symbionts: effectors interfering with plant immunity and establishing tolerance

In general, the mechanisms of defence of plants against pathogens involve numerous signals, starting with detection of pathogen-derived PAMPs and effectors molecules, followed by signal transduction from receptors to transcription factors, to the production of antimicrobial molecules and plant cell death.

There are effectors grouped for their roles as defensive effectors, in symbiotic bacteria, that interfere with some component of the plant immune system to protect the symbiosis, and offensive effectors that subvert some physiological functions of the plant for the benefit of the symbiont, i.e. to increase nutrient availability. Host physiological networks may trigger plant immunity and cause cell death while suppressing defence functions to promote nutrition. In addition, for the symbionts, it is necessary to avoid host cell death, while for a hemibiotroph apoptosis may be beneficial or undesirable, depending on the timing of the infection.

FLS2 in *Vitis vinifera*, VvFLS2 [77] differentially recognizes flagellin-derived epitopes from the endophytic growth promoting bacterium *Burkholderia phytofirmans* and plant pathogenic bacteria.

PTI can also lead to a reduction in type III-dependent effector protein translocation, suggesting that plants actively interfere with the expression of T3S genes and/or T3S-dependent protein delivery. It was postulated that mychorrizal fungi and bacteria promoting plant growth modulate the PTI signaling and are able to suppress chitin recognition in favour of the establishment of symbiosis [78-83].

Microbial decoys and bodyguards

Plant-fungi interactions, as well as plants with other invaders, have shown an evolutionary adaptation of hosts and invaders to produce enzymes and evolve new enzyme inhibitors. Among these products, are protein decoys and bodyguards, able to bind or mimic the targets of plant enzymes in order to act as a sponge. Microbial decoys are proteins mimicking the interaction domain of a protein partner, thus impeding its accessibility, or enzymes interfering with a plant defence mechanism [83-85]. Such decoys have been also named bodyguards, in that they are able to protect virulence factors from the action of resistance genes and plant defence pathways.

Glucanases are enzymes that degrade cell walls. *Botrytis cinerea* produces the family 11 xylanase that is blocked by the plant with production of endoxylanase inhibitors XIP-1 and TAXI-1 [48]. *Phytophtora sojae* secretes a xyloglucanase that damages soybean cell walls. Soybean, in turn, secretes a defense protein, Glucanase inhibitor protein-1 (GIP1) that binds endo β-1,3-glucanases. To counteract this plant defense, the oomycete deploys a secreted apoplastic xyloglucan-specific endoglucanase, PsXEG1, and the PsXEG1-like PsXLP1, that binds to GmGIP1 more tightly than does PsXEG1, an inactive enzyme that sequesters the plant inhibitor as a decoy, allowing the oomycete to invade the soybean cells. The gene pair encoding PsXEG1 and PsXLP1 is conserved in many *Phytophthora* species, and the *P. parasitica* orthologs PpXEG1 and PpXLP1 have similar functions [83-85]. The apoplastic decoy strategy may be widely used in *Phytophthora* pathosystems.

Chitin and the oligomers derived from the catabolism are sensed as molecular patterns. Fungi deacetylate the N-acetyl-glucosamine present in chitin in order to prevent its recognition as a PAMP. Furthermore, fungi have evolved protein decoys able to interfere with this recognition. Chitin hiding proteins thus antagonise and interfere with plant Chitin Binding Domain-chitinases. Finally, recent findings disclosed the role of pathogen bodyguards, proteins interfering with plant defence mechanisms, such as the mimicking Transcription Activator Like Effectors (TALE) [46], found in *Ralstonia solanacearum* and in Xanthomonads.

Transcription Activator-Like (TAL) effectors (TALE)

TAL effectors are able to activate expression of genes that induce plant defences. TALE proteins have a Nuclear Localisation Domain (NLS) and an acidic activation domain (AD), for the activation of the transcription machinery and expression of genes.

Xanthomonas AvrBs3 or TAL family infect more than 200 different plant families. According to their narrow host range, individual *Xanthomonas* strains are grouped into different pathovars (pv.). Some pathovars cause localised leaf spots and multiply extracellularly, within the leaf mesophyll or apoplast. In contrast, pathovars such as *Xanthomonas campestris* pv. *campestris* (*Xcc*) and *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) have access to the plant vascular system (xylem), spread systemically throughout the plant, and cause black rot or leaf blight disease [46]. Xanthan, released by Xanthomonas, blocks the xylem system causing wilting.

The pepper (*Capsicum annuum*) resistant cultivars Early Cal Wonder (ECW) carries the *Bs1* and *Bs3* dominant resistance (*R*) genes.

The TAL effectors AvrXa7, PthXo1, PthXo2, PthXo3 from *Xoo*, and PthA and PthB from *X. citri* pv. *citri* are major virulence determinants [46].

Os8N3/*Xa13* is a rice target gene induced by PthXo1 [46]. The recessive *xa13* allele acts as an *R* gene against *Xanthomonas* infections. Resistance is based on lack of PthXo1-mediated *Os8N3* expression in *xa13* homozygous plants.

A prototype of the resistance genes recognizing a TAL effector was *Xa27* [46]. *Xa27* is expressed only in resistant lines during *Xanthomonas* infection.
Thereafter, the pepper Bs3/avrBs3 dependent HR was studied [46]. Cloning of the *Bs3* gene from pepper resistant variety ECW-30R showed that *Bs3* expression and the HR depend on binding of AvrBs3 to a specific DNA element (*UPA* box) in the *Bs3* promoter. *Bs3* encodes a flavin monooxygenase (FMO). At least two additional *R* genes from rice (*Xa7*, *Xa10*) are under investigation. Bs4 is a TIR-NB-LRR protein that localizes to the plant cell cytoplasm, where it directs recognition of AvrBs4 TAL effector.
Nuclear Localisation Sequence (NLS) and Acidic Activation Domain (AD) in AvrBs3 are features typical of eukaryotic motifs, and are conserved: thus, AvrBs3 should have a functional role in plant cells. *R* genes detecting TAL effectors require the NLS and the AD in their sequence. For the mechanism of recognition, these molecular traps have been termed decoys.
Only a few effectors were shown to be major virulence factors because their deletion leads to a dramatic loss of virulence. AvrBs2 from the pepper and tomato pathogen *X. campestris* pv. *vesicatoria* strongly contributes to the multiplication of the bacteria in planta, while mutations in AvrXccC and XopXccN from *X. campestris* pv. *campestris* only weakly affect bacterial growth [46]. Several recent studies suggest that effectors of *Pseudomonas* and XopX from *X. campestris* pv. *vesicatoria* promote lesion development and growth in *Nicotiana benthamiana* through suppression of basal plant defense.
Interfering TALEs (iTALEs) are pathogen effectors able to overcome disease resistance [86]. In comparison with typical TALEs, iTALEs lack a transcription activation domain but retain nuclear localization motifs and are expressed from genes previously considered pseudogenes. The rice gene *Xa1*, encoding a nucleotide-binding leucine-rich repeat protein, was shown to confer resistance against *X. oryzae* isolates by recognizing multiple TALEs.
However, the presence of iTALEs in many isolates is able to interfere with the broad-spectrum resistance conferred by Xa1. Xa1 activates resistance, hypersensitive response (HR) and cell death, but this activation is suppressed by iTALEs expressed in Xoo and Xoc.

Plant signaling of defense activation using chemical priming.
The growth hormones jasmonic acid (JA); ethylene (ET), salicylic acid (SA); indole acetic acid (IAA); gibberellic acid (GA), activate signaling pathways that drive changes in gene expression, resulting in specific defense responses and induction of pathogenesis-related proteins.
Oxylipins, and in particular the lipoxygenase pathway leading to synthesis of JA, are well known regulators of the signaling pathways in response to biotic stresses, in some case overlapping with SA signaling [87-90]. Azelaic acid (AA) has been suggested to be a phloem-mobile signal that primes SA-induced defenses: its biosynthesis pathway is still unknown, being a derivative of oleic acid or its desaturated derivatives, linoleic and linolenic acids, through the activity of lipoxygenases and oxylipin synthesis genes.
Other plant secondary metabolites, such a nitric oxide (NO), contribute to the regulation of JA synthesis [88] and SA-dependent gene expression, including microRNAs [89].
Priming is related to compounds able to switch an activation state: during Induced Resistance (IR) response, plants react more rapidly to a stress because they are in an induced state. It was proposed to divide the priming phenomenon into three different stages: a priming phase, a post-challenge primed phase, and a trans-generational primed phase [92]. In this first stage, the levels of transcripts, proteins and metabolites are altered, with the plant in a standby state. In the post-challenge primed state, reactions fighting the stressor are induced rapidly. In the third phase,

plants generated from seeds of primed plants show a priming memory and react rapidly to pathogens.

Induced resistance (IR) leads to various types of systemic resistance throughout the plant. IR is based on two general mechanisms: direct activation of defense responses in systemic tissue after local stimuli and priming, which implies activation of systemic responses, but only when the pathogen reaches these sites. The best characterized type of IR is systemic-acquired resistance (SAR), which is mostly dependent on SA, unlike the less understood JA-dependent defense.

Cross-talk between different signaling pathways has been reported to generate both synergistic and antagonistic defense responses. In some cases this cross-talk might contribute to fine-tune defense responses against some pathogens according to its mode of infection.

Acibenzolar-S-methyl, or benzothiadiazole, is a functional analogue of SA hormone, that plays a central role in innate immunity as a co-activator of immunity-induced transcription reprogramming. Among the priming agents often used, are: methyl jasmonate, a volatile precursor of JA; beta amino butyric acid (BABA), that spread to leaves induces accumulation of SA, found important in defense against *P. syringae*; probenazole, inducing a general state of resistance: potassium phosphate, hexanoic acid, 2,6-dichloroisonicotinic acid and its methyl ester (both referred to as INA), and benzo (1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH), are priming agents which trigger SAR [93]. Resistance elicitors such as acibenzolar-S-methyl (ASM), cis-jasmone (CJ), β-amino butyric acid (BABA), which involve SA- and JA-dependent and independent signaling pathways, are widely used in field and in agriculture.

Activators or priming compounds approved in EU are: Acibenzolar-S-methyl (benzothiadiazole) and cerevisane [94]. Elicitors are plant activators with plant protection effect. Among those approved in EU, are: chitosans, fructose, heptamaloxylglucan, laminarin, pepino mosaic virus strain CH2 isolate 1906, Zucchini yellow mosaic virus strain 2020 [94-96]. A possible mechanism dependent on attenuated virus recognition has been proposed as LRR-receptor dependent [97].

COS-OGA is an elicitor made of an oligosaccharidic complex comprising chitooligosaccharides (COSs) and pectin-derived oligogalacturonides (OGAs) [98]. Therefore, it results from the association of both plant non-self PAMP (chitosan, with a mean polymerization degree of 7) and altered self molecules recognised as DAMP (oligopectates with a mean polymerization degree of 11). In plant immunity, OGAs are race-nonspecific elicitors that mimic degradation of plant cell wall and middle lamella pectin by fungal polygalacturonases [99].

Sclerotinia rot is fought using the biocontrol agents Contans, *Bacillus pumilus, Pythium oligandrum*, and *Trichoderma* spp., *Verticillum* spp. under development. *Fusarium* sp. in cereals, and late blight in potato, are fought using Polyversum (*Pythium oligandrum*), while *Pseudomonas* spp. and *Bacillus* spp. as antagonists are under development. Soil-borne pests (*Macrophomina*, sp. *Verticillium* sp., *Rhizoctonia* sp., *Plasmodiopora* sp., *Aphanomyces* sp., *Dickeya* sp., *Pectobacterium* sp., *Gaeumanomyces graminis)* are fought using Polyversum *(Pythium oligandrum*), *Trichoderma* spp., *Streptomyces* spp., while *Pseudomonas* spp. and *Bacillus* spp. as antagonists, under development. Powdery and downy mildew are fought using Green pesticides, induced resistance and plant resistance elicitors and antagonists. Laminarine (brown algae) is used as biocontrol for its effect as a DAMP signal. *Cydia pomanella*, pathogen of apple, pear and walnut, is fought using Granulosis virus and *Steinernema carpocapse* [94].

Although there are still studies under way to establish the potential for field application and crop protection [100-103], the exploitation of plant immune responses and SAR through improvement of transcription factor dependent gene expression is going to increase crop production. This may

combine with the identification of race-specific receptors and their introduction into susceptible varieties, to establish plant varieties with increased resistance to their pathogens.

Future perspectives and conclusions

There are several approaches possible to reinforce the immunity of plants to continuously evolving pathogen strains. The introgression of resistance genes has the main drawback that single *R* genes recognize only specific pathogen genotypes, whereas microbes can quickly loose effectors and evolve novel ones, thereby avoiding recognition. Previously, an effective and resistance against a broad spectrum of bacterial pathogens, was obtained by combining the Wall-associated kinase (WAK) ectodomain with the intracellular domain of FLS2 in tobacco [104, 105] and by transferring immune receptors among plant species, as reviewed in [106]. In ongoing research, FLS2 and EFR ectodomains were swapped with Cf9 intracellular domain, leading to enhanced activation of HR and necrotic lesions in tobacco (Unpublished results). It may be possible in the future, by exploiting novel techniques such as cisgenesis, to produce plants able to sense pathogen presence by a general PAMP and able to trigger an ETI response followed by HR. In the meantime, another strategy is to potentiate the plant surveillance system and phytohormone signaling leading to SAR by means of priming and chemicals already used in field. In addition, the activated state should not interfere with the normal plant development and the growth/defence trade off [30]. The main problems that we need to face is the differences existing among monocots and dicots, and especially the peculiar mechanisms present in Solanaceae that are not so easily transferred to other plant species. It is envisaged that in the future we will able to engineer olive trees with resistance to *Xylella fastidiosa*, tropical fruits with resistance to viruses, and to ensure the availability of food products ensuring food security despite the continuous appearance of novel pathogens and the transfer of new world pathogens to Europe and USA due to global trade of commodities.

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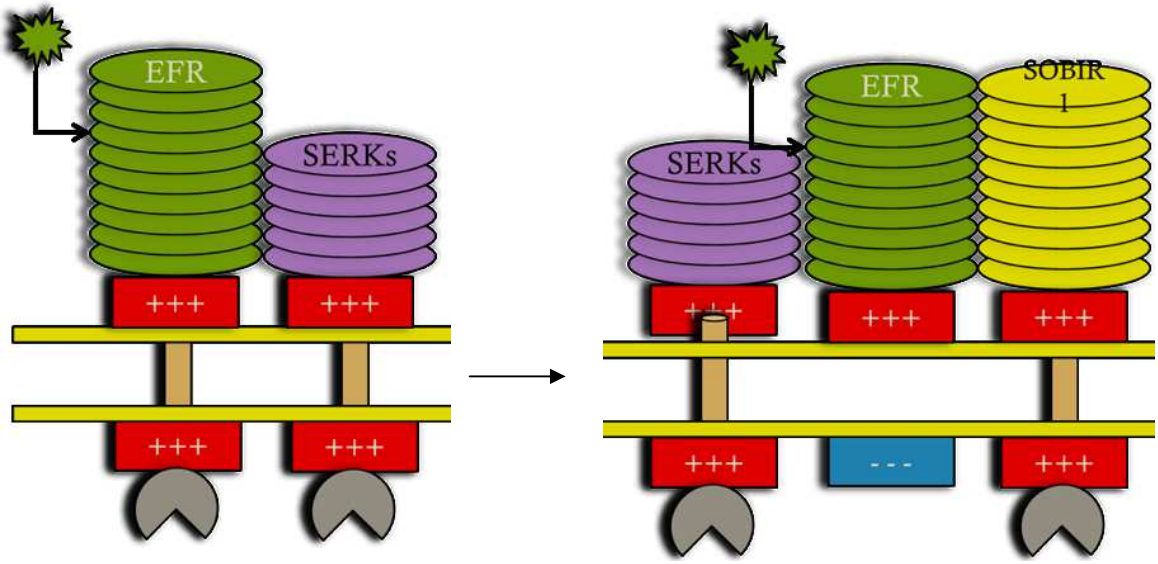
legends.

Figure 1. Formation of the LRR Receptor/co-repceptor kinase complex. EFR-SOBIR1-SERK complex and heterodimerization. After trans-phosphorylation beween the kinase domains, receptor endocytosis switches off the signal.



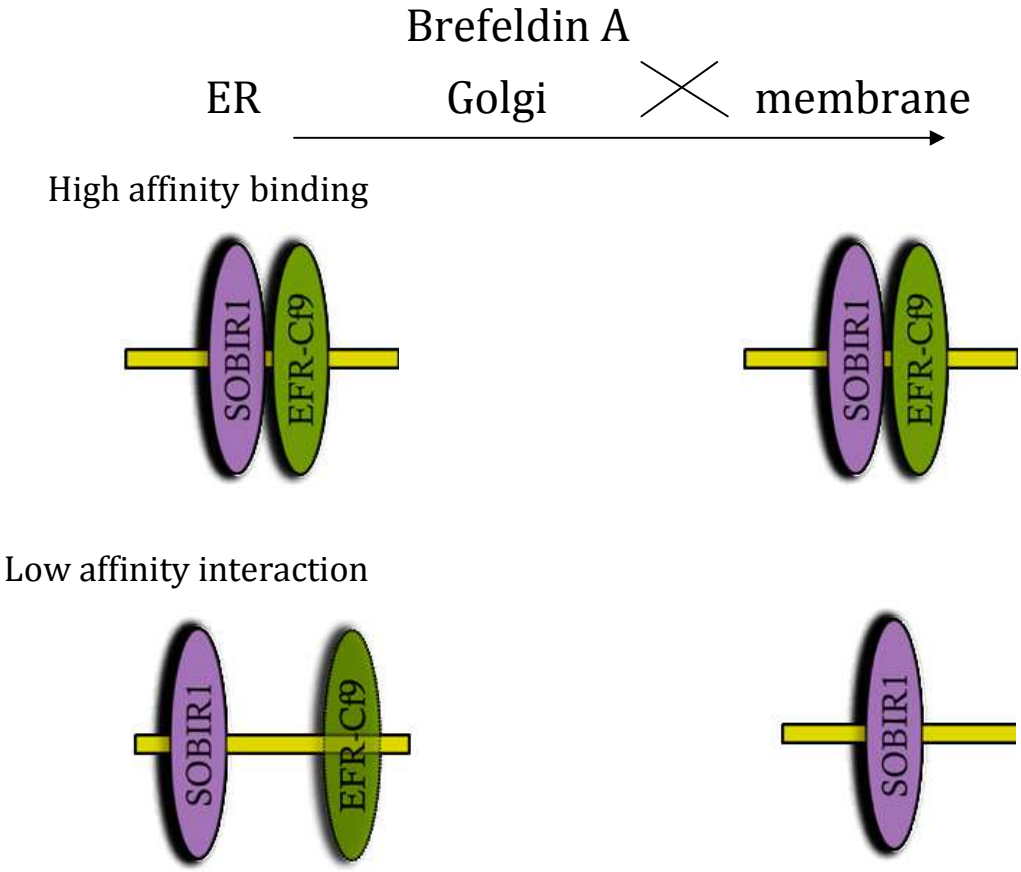


Figure 2. Activation of SOBIR1 and trans location. Protein folding by Endoplasmic Reticulum Quality Control (ERQC) system is needed for kinase domain co-receptors such as SOBIR1, cooperating with FLS2 and EFR, and with Cf9 protein (Receptor for *Cladosporium* Avr9) and Cf4 (for Avr4) in tomato. When brefeldin A, an inhibitor of translocation from ER to Golgi compartment and to plasma membrane, is added, the translocation to the membrane compartment does not occur. The formation of the complex with the LRR Receptor requires specific conditions, such as higher temperature and exposure for a minimum time before the creation of high affinity interaction.